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Chimeric West Nile/dengue virus vaccine candidate: Preclinical evaluation in mice, geese and monkeys for safety and immunogenicity

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Abstract

A live attenuated virus vaccine is being developed to protect against West Nile virus (WN) disease in humans. Previously, it was found that chimeric West Nile/dengue viruses (WN/DEN4 and WN/DEN4Δ30) bearing the membrane precursor and envelope protein genes of WN on a backbone of dengue type 4 virus (DEN4) with or without a deletion of 30 nucleotides (Δ30) in the 3′ noncoding region of the DEN4 part of the chimeric genome were attenuated and efficacious in mice and monkeys against WN challenge. Here, we report the generation of a clinical lot of WN/DEN4Δ30 virus and its further preclinical evaluation for safety and immunogenicity in mice, geese and monkeys. The vaccine candidate had lost neuroinvasiveness in highly sensitive immunodeficient mice inoculated intraperitoneally and had greatly reduced neurovirulence in suckling mice inoculated intracerebrally (IC). Compared to the wild-type WN parent, the chimeric virus was highly restricted in replication in both murine and human neuroblastoma cells as well as in brains of suckling mice. The WN/DEN4Δ30 virus failed to infect geese, indicating that chimerization of WN with DEN4 completely attenuated WN for this avian host. This observation suggests that the WN/DEN4 chimeric viruses would be restricted in their ability to be transmitted from vaccinees to domestic or wild birds. In monkeys, the WN/DEN4Δ30 vaccine candidate was highly immunogenic despite its low level of replication with undetectable viremia. Furthermore, the WN/DEN4Δ30 vaccine virus was safe and readily induced neutralizing antibodies against WN in monkeys immune to each of the four serotypes of dengue virus. These studies confirm the attenuation of WN/DEN4Δ30 for non-human primates, including dengue-immune monkeys, and demonstrate both a highly restricted replication (>10^8-fold decrease) in the brain of mice inoculated IC and an absence of infectivity for birds, findings that indicate this vaccine should be safe for both the recipient and the environment.

Keywords: West Nile virus; Live virus vaccine

1. Introduction

West Nile virus (WN) is a single-stranded, positive-sense RNA virus of the *Flaviviridae* family, and is a member of the Japanese encephalitis virus complex, an antigenically closely related group of mosquito-borne flaviviruses that also includes such important human pathogens as Japanese encephalitis, Murray Valley encephalitis, and St. Louis encephalitis viruses [1]. Similar to other members of this complex, WN is maintained in nature in a transmission cycle between mosquitoes and birds, with humans, horses, and other domestic and wild animals serving as incidental hosts. WN is endemic in Africa, Asia, Australia, and Europe, where it has usually been associated with mild human illness. Following its introduction in the Northeast region of the US in 1999, WN has spread rapidly across North America causing annual outbreaks and has recently reached Central and South

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America [2]. The emergence of this virus into a new environment has resulted in a significant increase in the fatality rate observed in humans, horses, and birds. During the 1999–2005 outbreaks of WN in the US, there were 19,381 human cases of WN illness reported that included 752 deaths [3], (CDC Reports: http://www.cdc.gov/ncidod/dvbid/westnile). Importantly, the severe neurological disease (meningitis or encephalitis) that required long-term rehabilitation was observed in over 30% of the confirmed WN cases and occurred with an increased frequency in the elderly and immunocompromised patients.

Several strategies have been pursued in the past years to develop effective vaccines to prevent WN disease [4]. However, only WN vaccines for veterinary use are commercially available in the US, and vaccine candidates for the prevention of WN disease in humans are still undergoing evaluation in pre-clinical or clinical trials [5–7]. Our leading vaccine candidate is a chimeric WN/DEN4Δ30 virus [8] which was created by replacing the membrane precursor (prM) and envelope glycoprotein (E) structural protein genes of the mosquito-borne dengue virus type 4 (DEN4) with the corresponding genes from WN strain NY99 and by introducing a 30 nucleotide deletion (Δ30) in the 3′ non-coding region of the DEN4 component of the chimeric genome. The resulting WN/DEN4Δ30 virus exhibited greatly reduced neurovirulence and neuroinvasiveness in Swiss Webster mice compared to its WN parent and was immunogenic providing complete protection against lethal WN challenge [8]. The experimental WN/DEN4Δ30 virus was also attenuated and immunogenic, and it provided protection in monkeys against wild-type WN challenge [5]. A clinical lot of this vaccine candidate virus was generated for use in humans and was further characterized in the present study for: (i) level of replication in the brain of newborn mice, (ii) neuroinvasiveness in immunodeficient mice, (iii) infectivity in the amplifying avian host, using young goslings as the avian model, and (iv) safety and immunogenicity in non-human primates with or without immunity to dengue viruses. Evaluation of the clinical lot of WN/DEN4Δ30 vaccine in human volunteers has begun.

2. Materials and methods

2.1. Cells and viruses

Simian *Vero* cells (World Health Organization seed, from passage 143 to 149) were maintained at 37 °C in an atmosphere of 5% CO_2 in OptiPRO SFM medium (Invitrogen, Carlsbad, CA) supplemented with 4 mM L-glutamine (Invitrogen) and 0.05 mg/ml of gentamicin (Invitrogen). Mouse Neuro-2A and simian LLC-MK_2 cells were purchased from the American Type Culture Collection and were maintained in MEM (Invitrogen) supplemented with 10% FBS and 1 mM L-glutamine. Human SH-SY5Y neuroblastoma cells were kindly provided by Dr. E. Dragunsky (CBER/FDA, Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS and 0.05 mg/ml of gentamicin.

The West Nile wild-type strain NY99 [8], dengue type 4 wild-type Caribbean strain 814,669 (wt DEN4) and its cDNA-derived recombinant DEN4(2A) and DEN4Δ30 [9] were prepared in *Vero* cells as described previously [8]. The previously described chimeric WN/DEN4 virus, that contained prM and E protein genes of WN, and its WN/DEN4-3′Δ30 mutant that contained a 30 nucleotide deletion in the 3′ noncoding region (NCR) of genome (referred to here as the WN/DEN4Δ30 experimental lot) were originally recovered after transfection of *Vero* cells with RNA transcripts of their full-length cDNA genomes [8]. Virus preparations of the unmodified WN/DEN4 and its Δ30 mutant had a titer of 1.7×10^6 and 6.4×10^5 PFU/ml, respectively. The experimental lot of WN/DEN4Δ30 virus was not recovered and passaged under current Good Manufacturing Procedures (cGMP) and therefore was not chosen for further vaccine development.

To generate a WN/DEN4Δ30 virus as a vaccine candidate for human use, the chimeric virus was independently rescued from a full-length cDNA (WN/DEN4-3′Δ30 clone 1) [8] and amplified in qualified simian *Vero* cells (WHO Seed, passage 145) under cGMP conditions. Subsequently, virus was biologically cloned by two successive passages at terminal dilution and finally amplified by three serum-free passages in qualified *Vero* cells. The final product, the WN/DEN4Δ30#1 lot of chimeric WN/DEN4-3′Δ30 virus (referred to here as the WN/DEN4Δ30 clinical lot) has a titer of 3.1×10^5 PFU/ml. Vaccine safety testing confirmed microbial sterility, tissue culture purity, and animal safety. The identity of the WN/DEN4Δ30 clinical lot virus was determined by complete sequence analysis of the genome.

The quantity of wild-type WN virus in cell-culture preparations, mouse brain suspension, or monkey serum was determined by titration on *Vero* cells using a plaque-forming assay (PFA) [8,10]. Confluent *Vero* cell monolayers on 12-well plates were inoculated in duplicate with serial ten-fold dilutions of virus, incubated for 2 days at 37 °C in an atmosphere of 5% CO_2 and immunostained using WN-specific antibodies in hyperimmune mouse ascitic fluid (HMAF) at a concentration of 1:1000 and conjugated goat anti-mouse immunoglobulins (Dako Co., Carpinteria, CA) at a concentration of 1:10. The chimeric WN/DEN4- or DEN4-infected cells in 24-well plates were incubated for 5 days and immunostained using either a DEN4-specific HMAF at a concentration of 1:1000 (for DEN4) or a 1:1 mixture of WN and DEN4 HMAF at a final concentration of 1:1000 (for the chimeric viruses).

2.2. Multi-cycle growth of chimeric viruses in mouse and human neuroblastoma cells

The kinetics and level of replication of parental and chimeric viruses were compared in murine Neuro-2A or in human SH-SY5Y cell lines. Cells grown on six-well

plates were inoculated with WN, DEN4, WN/DEN4, or WN/DEN4Δ30 (clinical lot) at a multiplicity of infection (MOI) of 0.01 PFU per cell and were allowed to adsorb for 1 h at 37 °C. Cell monolayers were washed twice in MEM, and 3 ml of fresh culture medium supplemented with 10% FBS was added. Virus in culture medium from one individual well was harvested daily, and the titer in each well was determined in *Vero* cells. Plaques were enumerated after 2 or 5 days of infection using the PFA described above.

2.3. Evaluation of chimeric viruses in mice

Chimeric and parental viruses were analyzed for peripheral virulence by the intraperitoneal (IP) inoculation of 3-week-old severe combined immunodeficient (SCID) mice in groups of 10 or 20. Female SCID mice (*ICRSC-M*; Taconic Farms, Germantown, NY) were inoculated with: (i) 10-fold serial dilutions of the WN NY99 virus ranging from 10^{-2} to 10^2 PFU; (ii) 10^6 PFU of DEN4; (iii) 10^3, 10^4, or 10^5 PFU of WN/DEN4 chimera; (iv) 10^2, 10^3, or 10^4 PFU of experimental or clinical lot of WN/DEN4Δ30; or (v) with MEM. Mice were observed for 49 days for encephalitis and moribund mice were humanely euthanized.

The neurovirulence of parental WN or rDEN4Δ30 and chimeric WN/DEN4Δ30 (experimental or clinical lot) was evaluated in suckling Swiss Webster mice (Taconic Farms) by intracerebral (IC) inoculation. Suckling mice (3 and 5 days of age) in groups of 9–12 were inoculated with a 10 μl volume of various concentrations of virus. Mice were observed for 21 days for encephalitis and moribund mice were euthanized humanely.

For study of virus replication in the mouse brain, 5-day-old Swiss Webster mice were inoculated IC with 10^3 PFU of the parental WN or DEN4, chimeric WN/DEN4, or its Δ30 deletion mutant (experimental or clinical lot) virus. The brains of four mice from each group were harvested daily from day 1 through day 5 and every other day from day 7 through day 19 after virus inoculation. Each mouse brain was homogenized individually to give a 30% suspension of phosphate-buffered Hanks' balanced salt solution (Invitrogen) supplemented with 7.5% sucrose, 5 mM sodium glutamate, 0.05 mg/ml of ciprofloxacin (Bayer HealthCare, West Haven, CT), 0.06 mg/ml of clindamycin (Pharmacia & Upjohn, Kalamazoo, MI), and 0.0025 mg/ml of amphotericin (Quality Biologicals, Gaithersburg, MD), and the brain suspension was clarified by low speed centrifugation. Subsequently the virus titer in each supernatant was determined in *Vero* cells using a PFA.

2.4. Evaluation of chimeric viruses in geese

Twenty two-week-old domestic geese (*Anser anser domesticus*) were screened for neutralizing antibody before the study was initiated and were found to be seronegative for WN and DEN4 as determined in the plaque-reduction neutralization (PRNT) tests described previously [8,11]. Groups of four goslings were inoculated subcutaneously (SC) in the nape of the neck with 10^3 PFU of wt WN or 10^4 PFU of wt DEN4 or chimeric virus and were observed for 21 days for WN-associated symptoms of disease such as decreased food or water intake, inactivity, weight loss (dehydration), fever, and neurological signs such as torticollis, drooping wing and head, tremor, convulsion, and paralysis [12]. Birds were bled daily for 5 days and on days 7 and 10 for detection of viremia and on day 21 for measurement of neutralizing antibody titer. The quantity of virus in bird serum was determined by direct titration on *Vero* cells using a PFA. Serum neutralizing antibody titer was determined by PRNT assay against wt WN [8,11]. On day 21 post-inoculation each of the surviving geese was challenged SC with 10^3 PFU of wild-type WN and observed daily during the following 10 days for development of neurological signs of disease. To determine viremia, blood samples were collected daily for 5 days and on days 7 and 10 post-challenge.

2.5. Studies with rhesus monkeys

2.5.1. Viremia and immunogenicity of the experimental and clinical lots of WN/DEN4Δ30 vaccine

The experimental and clinical lots of WN/DEN4Δ30 virus were evaluated in rhesus monkeys (*Macaca mulatta*) using previously established methods [5]. Groups of four monkeys, which were shown to be seronegative for WN and DEN4, were injected SC with 10^5 PFU of WN/DEN4Δ30 delivered in 1 ml of L-15 medium (Invitrogen). Serum was collected daily for 10 days to test for viremia and on days 28 and 42 for measurement of neutralizing antibody titer. Also, serum samples of two monkeys (monkey #13 and 14) from our previous study [5] that received a single 10^5 PFU dose of wt WN were reanalyzed in parallel for viremia and WN neutralizing antibody titer with those of the 8 monkeys that received experimental or clinical lot of WN/DEN4Δ30 in the present study.

2.5.2. Viremia and immunogenicity of chimeric WN/DEN4 viruses in monkeys previously immunized with dengue tetravalent vaccine

Two groups of rhesus monkeys were inoculated SC with 1 ml of an experimental, live-attenuated tetravalent dengue virus vaccine (TV-1 or TV-2 formulation) and then boosted with the same formulation on day 28 as described previously [13]. Specifically, (i) four monkeys in group 1 (Table 6) were immunized with TV-2 that contained 10^5 PFU of each recombinant rDEN1Δ30, rDEN4Δ30, and two antigenic chimeric viruses (rDEN2/4Δ30 and rDEN3/4Δ30), and (ii) another four monkeys in group 3 were immunized with TV-1 that contained 10^5 PFU/ml of each recombinant rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, and rDEN4Δ30 mutant. Ten months (313 days) after primary immunization with DEN tetravalent vaccine, these animals were bled on the day prior to chimeric WN/DEN4 inoculation and found to be seropositive for DEN4. Four monkeys in group 1 or 3

Table 1
Differences in nucleotide and amino acid sequences between experimental and clinical lots of WN/DEN4Δ30 vaccine candidate virus

Region of genome	Experimental lot		Clinical lot	
	Nucleotide position change	Amino acid change	Nucleotide position change	Amino acid change
E			$G_{1903}>A$	$Gly_{603} \rightarrow Arg$
NS2A			$A_{3484}>C$	$Gln_{1128} \rightarrow Pro$
NS3			$A_{4550}>C$	Silent
NS4B			$A_{7131}>G$	$Thr_{2344} \rightarrow Ala$
NS4B	$U_{7162}>C$	$Leu_{2354} \rightarrow Ser$		
NS4B			$U_{7474}>C$	$Val_{2458} \rightarrow Ala$

Note: Numbering of nucleotide sequence of the E protein gene derived from the sequence of the NY99 strain of WN (GenBank accession no. AR196835) and numbering of nucleotide (nt) sequence of nonstructural protein genes derived from sequence of DEN4Δ30 (clone p4Δ30) (GenBank accession no. AY376438). Numbering of amino acids in WN or DEN4 polyprotein begins with the methionine residue of the C protein (nt: 97–99 or 102–104, respectively) designated as residue 1.

with immunity to DEN4, together with a group of 4 naïve monkeys that served as unimmunized controls, were inoculated SC with 10^5 PFU of WN/DEN4 or the clinical lot of WN/DEN4Δ30, respectively. Serum was collected on days 1–10 and 42 after inoculation and stored at −70 °C. The quantity of virus in each serum sample from day 1 to day 10 was determined by direct titration on *Vero* cells using the immunostaining PFA, and serum neutralizing antibody titer was determined for samples collected on days 0 and 42 by PRNT assay against WN and DEN4.

3. Results

3.1. Sequence comparison of experimental and vaccine lots of WN/DEN4Δ30

Two versions of the chimeric WN/DEN4Δ30 virus, the experimental and clinical lots, were independently recovered from the same cDNA (clone 1) in *Vero* cells as described previously [8]. The nucleotide sequence of both viruses was determined and compared to that of the cDNA from which they were derived (Table 1). Since flaviviruses passaged in tissue culture cells develop mutations at high frequency [14–16], it was expected that the experimental and clinical lots of chimeric recombinant WN/DEN4Δ30 virus would develop independent mutations during their passage in *Vero* cells even though they were derived from the same plasmid DNA, and this did occur. Sequence analysis of the newly derived virus (clinical vaccine lot) revealed that during its recovery and amplification it accumulated four coding mutations located in E, NS2A, and NS4B, including an amino acid substitution $Thr_{2344} \rightarrow Ala$ in NS4B that was previously observed as a *Vero* cell adaptation mutation in DEN4 [17]. An amino acid substitution $Leu_{2354} \rightarrow Ser$ in NS4B that occurred in the experimental virus was not found in the newly generated clinical lot of WN/DEN4Δ30. The $Leu_{2354} \rightarrow Ser$ substitution in NS4B has been identified previously as a mutation that enhanced replication for DEN4 in *Vero* cells [15,17] and also was identified in the chimeric unmodified WN/DEN4 virus [8] and in the chimeric Langat/DEN4 or tick-borne encephalitis/DEN4 virus [16] following their passage in *Vero* cells. Thus, the two WN/DEN4Δ30 viruses differ in sequence, in part, by the acquisition of different *Vero* cell-adaptation mutations during passage in *Vero* cells. Because of this difference in sequence between the previously studied experimental lot of chimeric WN/DEN4Δ30 virus [5,8] and the newly produced clinical lot, it was necessary to determine if the mutations shared by the clinical and experimental lots of WN/DEN4Δ30 and not the differences in the sequences between the two preparations were primarily responsible for the desirable attributes of the vaccine candidate. Thus, attenuation of the virus for mice and monkeys was studied by comparing properties of the previously evaluated experimental lot with the newly generated clinical lot. In addition, the clinical lot was evaluated for infectivity for geese.

3.2. Attenuation of WN/DEN4 viruses in mice

3.2.1. Neuroinvasiveness of the chimeric viruses

Since the immunocompetent adult Swiss Webster mice were completely resistant to intraperitoneal (IP) inoculation with a large dose of the unmodified WN/DEN4 chimera or its Δ30 deletion mutant (experimental lot of WN/DEN4Δ30) as described previously [8], evaluation of the peripheral virulence of these viruses in the present study was performed only in highly sensitive SCID mice [18,19]. In a side-by-side comparison of the LD_{50}, the wild-type WN parent was extremely neuroinvasive for 3-week-old SCID mice with an IP LD_{50} of 0.8 $\log_{10}$ PFU (Table 2), whereas both the unmodified WN/DEN4 and its deletion mutant (experimental lot virus) had greatly reduced or ablated neuroinvasiveness when these chimeras were inoculated at the highest dose that could be tested (10^4 or 10^5 PFU). The lack of neuroinvasiveness of WN/DEN4 and WN/DEN4Δ30 chimeric viruses even in the immunodeficient mice indicates that both viruses are highly attenuated and that chimerization of WN with DEN4 most likely contributed to the observed decrease in neuroinvasiveness. The clinical lot of WN/DEN4Δ30, like the WN/DEN4Δ30 experimental lot, was also non-neuroinvasive in a separate, concurrent comparison in SCID mice (Table 2). These findings indicated that the WN/DEN4Δ30 candidate vaccine virus is highly restricted in its access to the CNS. Although the experimental

Table 2
Neuroinvasiveness of parental and chimeric viruses in 3-week-old SCID mice

Study	Mice inoculated IP with	Dose inoculated (PFU)	No. dead or moribund/no. tested (%)	LD_{50} (log_{10} PFU)
1	WN	0.01	0/20 (0)	0.8
		0.1	0/20 (0)	
		1	8/20 (40)	
		10	17/20 (85)	
		100	10/10 (100)	
	WN/DEN4	1000	0/20 (0)	>5.0[a]
		10000	0/20 (0)	
		100000	0/20 (0)	
	WN/DEN4Δ30 (experimental lot)	100	0/20 (0)	>4.0[a]
		1000	0/20 (0)	
		10000	0/20 (0)	
	DEN4	1000000	0/20 (0)	>6.0
2	WN/DEN4Δ30 (experimental lot)	10000	0/10 (0)	>4.0[a]
	WN/DEN4Δ30 (clinical lot)	10000	0/10 (0)	>4.0[a]

[a] Highest dose tested.

and clinical lots of WN/DEN4Δ30 virus differed in sequence (Table 1), the demonstration that each lot lacked neuroinvasiveness strongly suggests that the chimeric nature of the genome with its Δ30 mutation, and not their adventitious mutations, was responsible for the observed attenuation phenotype of both lots of WN/DEN4Δ30.

3.2.2. *Neurovirulence of WN/DEN4Δ30 in mice*

The parental (WN and rDEN4Δ30) viruses and their WN/DEN4Δ30 chimera (clinical lot) were evaluated in parallel in 5-day-old Swiss mice by intracerebral (IC) inoculation of different quantities of virus by measuring morbidity and mortality as the outcome (Table 3). Wild-type WN was highly neurovirulent in 5-day-old Swiss mice with an IC LD_{50} of 0.5 log_{10} PFU. Recombinant DEN4Δ30 virus that served as the genetic background for construction of WN/DEN4Δ30 chimera was much less neurovirulent with an IC LD_{50} of 4.5 log_{10} PFU. All of the mice inoculated with 10 or 10^3 PFU of WN/DEN4Δ30 survived during a 21-day observation period. At a dose of 10^2 PFU, only 2 of 11 mice inoculated with this chimera died. The interval of time to death of the two mice inoculated with the clinical lot of WN/DEN4Δ30 chimera was twice as long as that of mice inoculated with WN. Thus, the WN/DEN4Δ30 chimera (clinical lot) more closely resembled the low neurovirulence phenotype of its rDEN4Δ30 parent rather than the high neurovirulence of the WN parent, but retained a low level of neurovirulence. In an additional comparison of the experimental and clinical lots of WN/DEN4Δ30 in younger suckling mice (3-day-old Swiss mice, Table 3), 2 of the 10 suckling mice inoculated IC with 10^3 PFU of the clinical lot of WN/DEN4Δ30 virus died, and no morbidity or lethality was observed in mice inoculated with the same dose of the experimental lot of virus. Thus, both experimental and clinical lots of WN/DEN4Δ30 were

Table 3
Neurovirulence of parental and chimeric viruses in 3- or 5-day-old Swiss mice

Study	Mice inoculated IC with	Age of mice	Dose inoculated (PFU)	No. dead or moribund/No. tested (%)	LD_{50} (log_{10} PFU)
1	WN	5-day-old	0.01	0/11 (0)	0.5
			0.1	0/10 (0)	
			1	1/10 (10)	
			10	11/11 (100)	
	rDEN4Δ30		100	1/10 (10)	4.5
			1000	2/10 (20)	
			10000	4/11 (36)	
			100000	5/11 (45)	
	WN/DEN4Δ30 (clinical lot)		10	0/10 (0)	>3.0
			100	2/11 (18)	
			1000	0/10 (0)	
	Control			0/12 (0)	
2	WN	3-day-old	1	9/9 (100)	−0.5[b]
	WN/DEN4		10000	5/10 (50)	∼4.0[b]
	WN/DEN4Δ30 (experimental lot)		1000	0/10 (0)	>3.0[a]
	WN/DEN4Δ30 (clinical lot)		1000	2/10 (0)	>3.0[a]

[a] Highest dose tested.
[b] Data from previous studies presented for purpose of comparison [8].

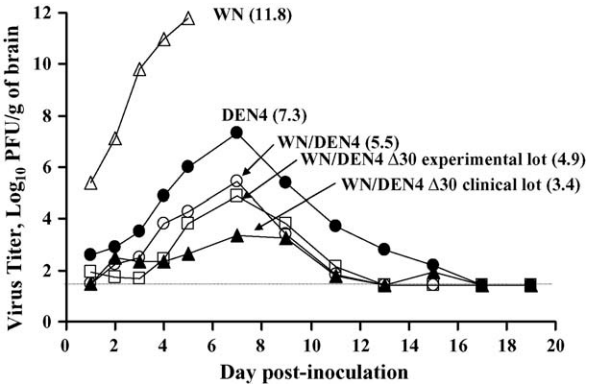

Fig. 1. Replication of parental and chimeric viruses in mouse brain. Five-day-old Swiss mice were inoculated IC with 10^3 PFU of virus. At indicated days, brains of four mice were harvested and virus titer of each individual brain suspension was determined by plaque assay in *Vero* cells. Mean peak virus titer ($\log_{10}$ PFU/g of brain) is shown in parentheses.

attenuated and less neurovirulent in 3-day-old mice than their wild-type WN or DEN4 parent which had an IC LD_{50} of -0.5 or $2.6\log_{10}$ PFU, respectively, as estimated previously for this age of mice [8].

3.2.3. Replication of viruses in mouse brain

The increase in IC LD_{50} of the WN/DEN4 and WN/DEN4Δ30 chimeras over that of the WN virus suggested that the chimeras were restricted in replication in mouse brain compared to that of their WN parent. In order to quantitatively estimate the level of attenuation specified by chimerization of WN with DEN4 and, separately, by the addition of the Δ30 mutation to the WN/DEN4 virus, the parental and chimeric viruses were evaluated for their ability to replicate in the brain of suckling mice. Following direct IC inoculation of 5-day-old Swiss mice with 10^3 PFU of virus, brains of mice were harvested, and virus titer of individual brain suspension was determined. Resulting mean titers are shown in Fig. 1. WN replicates efficiently in the brain of mice and attains a titer of $11.8\log_{10}$ PFU/g of brain 5 days after inoculation. At this dose of WN, mice developed symptoms of encephalitis or died on day 6 post-inoculation. Wild-type DEN4 also replicates efficiently, and its peak titer of $7.3\log_{10}$ PFU/g of brain was observed 7 days after IC inoculation. The unmodified WN/DEN4 chimera reached a peak virus titer of $5.5\log_{10}$ PFU/g of brain, indicating that WN/DEN4 replicated less well in mouse brain than either WN or DEN4 parental virus and that the chimerization of WN with DEN4 was the major determinant of the observed attenuation of WN/DEN4.

The WN/DEN4Δ30 virus was slightly more restricted in replication in mouse brain than the unmodified WN/DEN4 chimera. On day 7, the experimental and clinical lots of WN/DEN4Δ30 vaccine attained titers of 4.9 and $3.4\log_{10}$ PFU/g of brain, respectively. This represented an additional 4- and 125-fold decrease in replication of Δ30 mutants compared to their unmodified WN/DEN4 parent, indicating that the Δ30 mutation independently attenuates WN/DEN4 for mice. These findings clearly indicate that chimerization and the presence of the Δ30 mutation dramatically decreased replication of the clinical lot of WN/DEN4Δ30 compared to its highly virulent WN parent, a fold reduction that was greater than 10^8. Overall, both greatly decreased lethality and greatly restricted replication in the brain following direct IC inoculation indicate that the clinical lot of WN/DEN4Δ30 is highly attenuated for the CNS of mice.

3.3. In vitro replication of chimeric viruses in murine and human cells of neural origin

The level of replication of the WN/DEN4 viruses was evaluated in murine and human neuroblastoma cells to determine if there is a correlation between *in vitro* replication in cells of neural origin and the attenuation of virus *in vivo* as demonstrated previously for attenuated mutants of Langat virus (LGT) [20], its LGT/DEN4 chimera [16], or poliovirus [21,22]. For this reason, the kinetics of replication of the WN/DEN4 chimera or the Δ30 deletion mutant (clinical lot) were compared to those of the parental WN and DEN4 viruses by infecting murine Neuro-2A or human SH-SY5Y neuroblastoma cells at a MOI of 0.01 PFU/cell and measuring virus yield at 24-h intervals (Fig. 2). WN replicated efficiently in

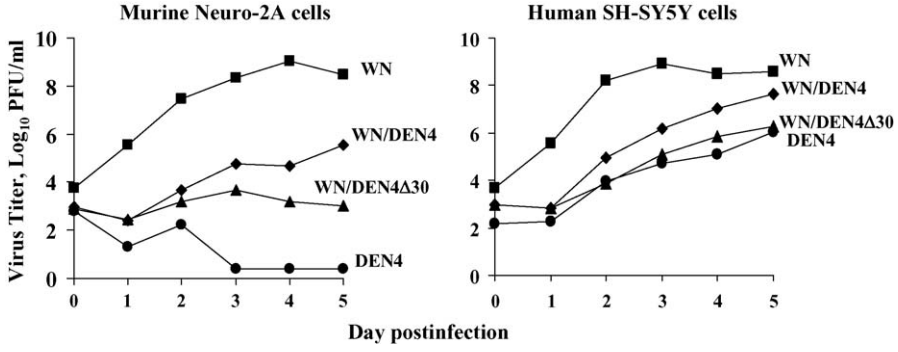

Fig. 2. Analysis of growth of parental WN or DEN4 and their chimeric WN/DEN4 virus or clinical lot of the WN/DEN4Δ30 mutant in mouse Neuro-2A and human SH-SY5Y neuroblastoma cells. Cells were infected with the indicated virus at MOI of 0.01, and the titer of virus in culture medium harvested at indicated times was determined in *Vero* cells.

Table 4
WN/DEN4 chimeric viruses are non-infectious in geese

Virus	Viremia after inoculation[a]			Mean serum neutralizing antibody titer against WN[c]	Viremia after WN challenge[b]		
	No. with viremia	Mean no. days	Mean peak virus titer (log_{10} PFU/ml)		No. geese viremic	Mean no. days	Mean peak virus titer (log_{10} PFU/ml)
WN	4/4	5.5	5.4	264	0/2	0	<0.7
DEN4	0/4	0	<0.7	<20	4/4	3.8	5.0
WN/DEN4	0/4	0	<0.7	<20	4/4	3.3	4.4
WN/DEN4Δ30 (clinical lot)	0/4	0	<0.7	<20	4/4	3.5	4.8
Control				<20	4/4	3.5	4.3

[a] Two-week-old goslings were inoculated in the nape of neck with 10^3 PFU of WN or with 10^4 PFU of DEN4 or chimeric virus. Two of the four young goslings that received the wild-type WN virus developed severe neurologic disease and were euthanized on day 6 or 8 post-inoculation.
[b] Twenty-one days after inoculation, the surviving geese were challenged SC with 10^3 PFU of WN.
[c] Plaque reduction (60%) neutralizing antibody titer 21 days after primary inoculation. Reciprocal titers are shown.

both cell lines with virus titers reaching 8.5–8.6 log_{10} PFU/ml on day 5 post-infection. In contrast, DEN4 replicated less efficiently in human SH-SY5Y cells attaining a titer that was 300 times lower than that of WN, and its growth in murine neuroblastoma cells was even more restricted. Chimerization of WN with DEN4 reduced replicative capacity of the resulting WN/DEN4 virus by 1000-fold in murine Neuro-2A and 20-fold in human SH-SY5Y cells, respectively, when compared with its WN parent. In addition, a 300- or 20-fold decrease in replication of the WN/DEN4Δ30 mutant compared to its unmodified WN/DEN4 parent virus was observed in murine or human neuroblastoma cell culture, respectively. Thus, our findings indicate that both the chimerization of WN with DEN4 and the Δ30 mutation independently attenuate WN/DEN4Δ30 virus for growth *in vitro* in the neuroblastoma cell cultures and *in vivo* in the CNS of mice.

3.4. Restricted replication of chimeric viruses in domestic geese

WN is maintained in nature in a mosquito-bird-mosquito cycle involving many species of birds that serve as primary vertebrate reservoir hosts [23,24]. Thus, it was important to evaluate the ability of WN/DEN4 chimeric viruses to replicate in birds since it is theoretically possible that a WN/DEN4 chimeric vaccine virus could be transmitted from a vaccinee to a bird by a mosquito. Geese (*Anser anser domesticus*), which served as a model for domestic poultry in this study, are highly susceptible to natural or experimental WN infection and develop long-lasting viremia and neurological disease or death [12,25]. When 2-week-old geese were infected with 10^3 PFU of WN, three of the four goslings developed clinical signs, including decreased activity, reluctance to rise upon stimulation, elevated body temperature, dehydration, head tremor, partially closed eyes, and head in lower posture. Two of these moribund goslings were euthanized on days 6 and 8 post-infection because of persistent neurological signs. Each of the four WN-infected birds developed viremia that lasted 5 or 6 days (Table 4) and achieved a virus titer in serum between 4.2 and 6.5 log_{10} PFU/ml (mean = 5.4 log_{10} PFU/ml). In contrast, viremia was not detected in young geese that received 10^4 PFU of the DEN4 parent, WN/DEN4 chimera, or WN/DEN4Δ30 clinical lot virus, indicating that these viruses did not replicate in highly susceptible geese. In addition, none of the goslings that received the WN/DEN4 chimera or its deletion mutant developed neutralizing antibodies to WN on day 21 post-inoculation (Table 4). On day 21 post-inoculation, two geese that survived the primary WN infection developed a high level of WN neutralizing antibodies which was sufficient to prevent viremia, clinical signs, and death after challenge with WN. In contrast, each of the geese that received DEN4 or a WN/DEN4 chimeric virus, as well as the four naïve control birds, became viremic after challenge with WN. The duration of viremia and mean peak WN virus titer in serum following WN challenge did not differ between vaccinated and unvaccinated geese. Although none of the geese died from the WN challenge, three of the four birds in each group developed clinical signs of WN disease during 10 days post-challenge observation which included uncoordinated gait, drooping wings, imbalance while standing, head held in lowered posture, recumbency, torticollis, head shaking, and rhythmic side-to-side head movement. The high morbidity rates and high level of viremia in the vaccinated and non-vaccinated geese indicate that the WN/DEN4 chimeras failed to stimulate protective immunity against WN in the domestic geese. Thus, the responses to immunization and challenge with WN indicate that both chimeras failed to infect and replicate in geese. The lack of infectivity of the WN/DEN4Δ30 virus for geese suggests that its use in humans or other mammals as a vaccine poses no threat to wild or domestic birds. Unfortunately, it also precludes its use as a vaccine in birds.

3.5. Comparison of the safety and immunogenicity of the experimental and clinical lots of the WN/DEN4Δ30 vaccine in rhesus monkeys

Safety, immunogenicity, and protective efficacy of the WN/DEN4 and WN/DEN4Δ30 (experimental lot) chimeric viruses were evaluated previously in rhesus monkeys at a

Table 5
Response of rhesus monkeys immunized with 10^5 PFU of the clinical or experimental lot of WN/DEN4Δ30 chimeric virus

Immunizing virus	Monkey number	Viremia (log_{10} PFU/ml) on indicated day post-immunization								Serum neutralizing antibody titer on indicated day[a]	
		0	1	2	3	4	5	6	7	28	42
WN/DEN4Δ30 (experimental lot)	CJ34	–	–	–	–	–	–	–	–	328	102
	CJ9C	–	–	–	–	–	–	–	–	287	108
	CK2H	–	–	–	–	–	–	–	–	325	158
	CK6F	–	–	–	–	–	–	–	–	325	81
		–	–	–	–	–	–	–	–	GMT: 316	109
WN/DEN4Δ30 (clinical lot)	CK1D	–	–	–	–	–	–	–	–	284	106
	CK55	–	–	–	–	–	–	–	–	303	141
	CK4X	–	–	–	–	–	–	–	–	365	271
	CK2J	–	–	–	–	–	–	–	–	351	205
										GMT: 324	170
WN[b]	13	–	–	2.2	2.3	2.2	2.2	1.8	–	2131	
	14	–	0.7	2.8	2.3	2.1	2.3	–	–	1987	
										GMT: 2059	

[a] Plaque reduction (60%) neutralizing antibody titer was determined against WN NY99 using serum collected on indicated day post-immunization. Reciprocal titers are shown. Geometric mean titers (GMT) are calculated for each group.

[b] The serum samples are historical samples from our previous study collected from two monkeys injected SC with 10^5 PFU of wt WN NY99 [5]. These samples were retested for measurement of viremia or WN-specific neutralizing antibody titer in parallel with those of the eight monkeys that received the experimental or clinical lot of WN/DEN4Δ30.

dose of 10^5 or 10^6 PFU, and the results of this study have been published [5]. Despite the high level of attenuation, the experimental WN/DEN4Δ30 vaccine virus induced a high titer of WN-specific neutralizing antibodies and provided complete protection in monkeys against challenge with wt WN. In the present study, the level of attenuation and immunogenicity of the WN/DEN4Δ30 clinical lot was determined in rhesus monkeys in a concurrent comparison with the experimental lot. The findings summarized in Table 5 demonstrate that both the experimental and clinical lots of vaccine candidate exhibited highly restricted replication, i.e., none of the four monkeys inoculated with 10^5 PFU of either experimental or clinical lot of WN/DEN4Δ30 virus developed detectable viremia ($<10^{0.7}$ PFU/ml). Also, clinical illness was not seen in any monkey. Although both chimeric viruses were significantly attenuated for monkeys, they induced a high level of serum WN neutralizing antibodies in each immunized animal, and the immune response for these two lots was comparable. It was shown previously [5] that a single dose of 10^5 PFU of the experimental lot was highly efficacious in rhesus monkeys against wt WN challenge, and this was not evaluated in the present study with the clinical lot of WN/DEN4Δ30 since both vaccine viruses exhibited comparable levels of immunogenicity.

3.6. Evaluation of the chimeric viruses in DEN-immune monkeys

Since chimeric WN vaccine candidates (WN/DEN4 and WN/DEN4Δ30) were constructed on the backbone of DEN4, it was important to determine whether pre-existing immunity to the dengue viruses would affect the level of replication (viremia) or immunogenicity of WN/DEN4 viruses in non-human primates. Two groups of rhesus monkeys that received two doses of live-attenuated tetravalent dengue virus vaccine (TV-1 or TV-2 formulation; see Materials and Methods) at a 1 month interval [13] were used as animals with pre-existing DEN-immunity (monkeys in groups 1 and 3; Table 6). Each monkey in these two groups was seropositive to DEN4 on the day prior to WN vaccine immunization with DEN4-specific neutralization titers ranging from 1:26 to 1:328. In contrast, the low level of WN-specific cross-reactive neutralizing antibodies (1:13) was found only in the group 3 of monkeys previously immunized with the TV-1 formulation.

DEN-immune monkeys and naïve monkeys were inoculated subcutaneously with a single dose of 10^5 PFU of the chimeric WN/DEN4 virus or its Δ30 mutant (Table 6). The interval between the primary DEN vaccine inoculation and the WN vaccine administration was 10 months. WN/DEN4 induced only a brief viremia in one of four DEN-immune and in two of four non-immune monkeys. Viremia lasted 1 or 2 days and attained a low level that was comparable for these two groups of monkeys. In contrast, none of the four naïve or DEN-immune monkeys inoculated with 10^5 PFU of the clinical lot of the WN/DEN4Δ30 vaccine developed a detectable viremia. These observations demonstrate that there was no difference in the level of replication of the WN/DEN4 virus or its Δ30 mutant between DEN-immune and non-immune monkeys, i.e., enhancement or interference in replication of chimeric WN/DEN4 viruses in DEN-immune monkeys had not occurred. Each chimeric virus was similarly attenuated for monkeys with or without immunity to DEN.

Following WN/DEN4 or WN/DEN4Δ30 inoculation, all animals seroconverted to WN, as determined by the presence of WN-specific neutralizing antibodies in serum on day 42 post-immunization (Table 6). At that time, the level of neu-

Table 6
Effect of previous DEN immunity on infectivity and immunogenicity of WN/DEN4 chimeric vaccines in rhesus monkeys

Group	Monkey ID #	Prior DEN immunity at time of WN/DEN4 immunization[a]	Serum neutralizing antibody titer[b] on day prior to WN/DEN4 immunization		WN vaccine immunization								
					Immunizing virus	Viremia (log_{10}PFU/ml) on indicated day following inoculation with WN vaccine virus[c]							Serum neutralizing antibody titer against indicated virus[d]	
			αDEN4	αWN		1	2	3	4	5	6	7	αDEN4	αWN
1	CJ76	Yes	43	<10	WN/DEN4	–	–	–	–	–	–	–	228	618
	CL51		26	<10		–	–	–	–	–	–	–	270	834
	CK5J		116	<10		–	–	–	–	–	–	–	659	620
	CK6D		160	<10		–	–	–	–	0.7	–	–	386	842
			GMT: 67	<10									354	720
2	CL8G	No	<10	<10	WN/DEN4	–	–	–	–	0.7	–	0.7	<10	3140
	CL37		<10	<10		–	–	–	–	–	–	–	<10	1334
	CL19		<10	<10		–	–	–	–	–	–	1.0	<10	1900
	CL17		<10	<10		–	–	–	–	–	–	–	<10	596
			GMT: <10	<10									<10	1476
3	CK1C	Yes	328	13	WN/DEN4Δ30	–	–	–	–	–	–	–	328	108
	CL2V		210	13	(clinical lot)	–	–	–	–	–	–	–	367	128
	CK1H		69	14		–	–	–	–	–	–	–	353	312
	CJ9H		82	13		–	–	–	–	–	–	–	188	260
			GMT: 141	13									299	183
4	CL4G	No	<10	<10	WN/DEN4Δ30	–	–	–	–	–	–	–	<10	144
	DA2G		<10	<10	(clinical lot)	–	–	–	–	–	–	–	<10	604
	CL2X		<10	<10		–	–	–	–	–	–	–	<10	292
	CK8J		<10	<10		–	–	–	–	–	–	–	<10	648
			GMT: <10	<10									<10	398

[a] Rhesus monkeys in group of 4 were inoculated SC with tetravalent dengue vaccine (TV-1 or TV-2 formulation as specified in Section 2) in a 1-ml dose and boosted on day 28 as described previously [13].
[b] Plaque reduction (60%) neutralizing antibody titers ($PRNT_{60}$) were determined against wild-type WN or DEN4 virus. Serum for neutralization assay was collected on day 0 (313 days after first immunization with DEN vaccine). Reciprocal titers are shown. Geometric mean titers (GMT) are calculated for each group.
[c] Serum used to measure viremia was collected daily for 10 days. Virus titer in serum was determined by plaque-forming assay on *Vero* cells, viremia was not detected in any monkey after day 7 post-inoculation. The lower limit of detection was 0.7 log_{10} PFU/ml.
[d] Serum for $PRNT_{60}$ was collected on day 42 post-immunization with WN vaccine. Reciprocal titers are shown.

tralizing antibodies to DEN4 was boosted (defined as a two-fold or greater increase in antibody titer) in serum of monkeys that previously received two doses of the tetravalent DEN vaccine. The WN/DEN4 and WN/DEN4Δ30 chimeric viruses induced a comparable, high level of serum WN neutralizing antibodies (range of 1:108–1:3140) in DEN-immune or naïve animals. However, the geometric mean WN neutralizing antibody titer induced by WN/DEN4 or its Δ30 mutant in DEN-immune monkeys was slightly lower (approximately two-fold) than that induced in monkeys without immunity to DEN, but group sizes were too small to assess the significance of this observation. The WN-specific neutralizing antibody titer in the serum of monkeys (DEN-immune or non-immune group) that received WN/DEN4Δ30 was approximately four-fold lower than that of monkeys infected with a comparable dose of the WN/DEN4 virus, a finding consistent with the greater attenuation of the WN/DEN4Δ30 vaccine candidate. This was also observed previously in a comparison of the experimental lot of WN/DEN4Δ30 vaccine with the WN/DEN4 virus [5].

4. Discussion

Previous studies of WN/DEN4 and WN/DEN4Δ30 in Swiss Webster mice [8] demonstrated that both chimeras are highly attenuated and efficacious in immunocompetent mice, i.e., they exhibited greatly reduced neurovirulence, lacked neuroinvasiveness, and provided complete protection of mice against WN challenge. Chimerization of WN and DEN4 was the major factor that led to the satisfactory balance between attenuation and immunogenicity of WN/DEN4 for mice. An independent contribution of the Δ30 mutation to attenuation of WN/DEN4Δ30 virus in mice was not apparent. However, in rhesus monkeys [5], WN/DEN4Δ30 virus was found to be more attenuated than its WN/DEN4 progenitor, strongly suggesting that both chimerization and the genetically stable Δ30 deletion mutation independently contribute to attenuation of WN/DEN4Δ30 virus. Importantly, the more attenuated vaccine candidate, WN/DEN4Δ30, induced a high titer of neutralizing antibodies and prevented viremia in monkeys challenged with WN. In the current study, we generated a second lot of WN/DEN4Δ30, designated the clinical lot, for evaluation as a WN candidate vaccine in humans. The previously tested experimental lot and the clinical lot had a different set of adventitious mutations (Table 1). It is possible that these adventitious mutations could have contributed differently to the properties of the WN/DEN4Δ30 viruses in mice or monkeys. However, since the level of attenuation of the experimental and clinical lot for mice and monkeys was comparable, it can be concluded that both chimerization and the Δ30 mutation, but not the presence of the adventitious mutations, are the major determinants of attenuation of WN/DEN4Δ30 virus for mice and non-human primates. Also, these observations extend previous findings demonstrating that the Δ30 deletion mutation attenuated two dengue viruses (DEN1Δ30 and DEN4Δ30) [9,26] and the chimeric tick-borne encephalitis/DEN4Δ30 flavivirus [16] for mice, monkeys, or humans. The Δ30 mutation appears to be a very effective mutation for developing live attenuated flavivirus vaccines.

The previous study of WN/DEN4Δ30 in mice was extended in the present study, and the WN/DEN4Δ30 vaccine was found: (i) to lack neuroinvasiveness for highly sensitive immunodeficient mice and (ii) to be less neurovirulent than its WN parent in suckling mice. It retained a low level of neurovirulence, but the level was even less than that of its rDEN4Δ30 parent, a virus that is highly attenuated in humans [27]. The decreased neuroinvasiveness and neurovirulence of WN/DEN4 and WN/DEN4Δ30 for mice suggested that both viruses were restricted in replication in the CNS of mice. The WN/DEN4 chimera was remarkably decreased in replication in the brains of newborn mice compared to that of its WN (by a factor of 10^6) and DEN4 (by a factor of $10^{1.8}$) parents (Fig. 1). Replication of WN/DEN4Δ30 vaccine virus was an additional 4- to 125-times more restricted than that of the unmodified WN/DEN4 chimera. These findings provided strong evidence that both chimerization and Δ30 mutation independently contribute to reduction of WN/DEN4Δ30 vaccine virus replication in the brain of mice. These new findings may have important implications for the safety of WN/DEN4Δ30 vaccine for humans. The greatly reduced neuroinvasiveness predicts that the vaccine virus should be greatly restricted in spread from the peripheral site of inoculation to the CNS of the vaccinee. The greatly reduced replication in the CNS of mice predicts that if the WN/DEN4Δ30 virus enters the CNS of a vaccinee, it should be restricted in replication and highly attenuated for that site. Thus, these two properties, decreased neuroinvasiveness and decreased replication in the CNS, should increase the safety of this vaccine in humans.

Interestingly, chimerization of WN with DEN4 and the addition of Δ30 mutation resulted in a decrease in virus replication in both murine and human neuroblastoma cells (Fig. 2). With an exception for DEN4, there was a correlation between reduction of *in vitro* replication in cells of neural origin and the restricted replication in mouse brain of the chimeric viruses, notably, the more attenuated WN/DEN4Δ30 virus in mice had lower replicative capacity in murine or human neuroblastoma cells compared to that of its immediate parental WN/DEN4 or WN progenitor. It should be noted that findings in the present study with chimeric WN/DEN4 viruses and those in previous studies with a non-neuroinvasive tick-borne Langat virus mutant [20] or with chimeric LGT/DEN4 virus [16,20] correlate the restriction of replication in cells of neuronal origin with attenuation in mice and monkeys. This correlation suggests that a decrease in replication of neurotropic virus in neuroblastoma cells, which is also reflected in its restricted replication in mouse brain tissue, might serve as a predictor of *in vivo* attenuation due to a common mechanism of attenuation and might be useful in the development of a live attenuated vaccine candidate against viruses causing neurological disease.

To preclude introduction of a vaccine virus into a natural transmission cycle, a live WN vaccine virus should have low potential for both mosquito transmission and decreased capacity for replication in birds as amplifying hosts. In a previous study [28], we have shown that both the WN/DEN4 virus and the clinical lot of WN/DEN4Δ30 virus were unable to generate disseminated infections in two mosquito vectors: *Culex tarsalis*, a vector species competent for WN, and *Aedes aegypti*, which is the primary vector of dengue viruses. Also, *Aedes albopictus*, a mosquito species susceptible to both DEN4 and WN virus in nature, retained a moderate level of susceptibility to chimeric viruses, but the disseminated infections produced by the chimeric viruses failed to pass into the mosquito saliva. These observations suggest that chimerization of two mosquito-borne flaviviruses, WN and DEN4, acted to restrict infectivity of the hybrid viruses for *Culex* and *Aedes* mosquitoes, limiting the potential of these viruses for transmission by the most permissive mosquito vectors tested in our study. Thus, both the greatly reduced replication in nonhuman primates (low level or undetectable viremia) and the decreased infectivity for mosquitoes suggest that chimeric viruses would be greatly impaired in their transmission from vaccinees to other hosts via a mosquito vector.

Since both attenuated WN/DEN4 and its Δ30 mutant are being considered as novel vaccine candidates for veterinary and human use, studies in geese were necessary to evaluate whether the viruses could be (i) transmitted from a vaccinee to an avian host in the environment and (ii) used as a vaccine for birds. In the present study, young domestic geese, which serve as a model for domestic poultry or wild birds and are highly permissive to WN infection, were infected with WN or the chimeric viruses. Three of the four WN-infected goslings became moribund and two of them were euthanized due to the signs of severe neurologic disease. Each of the WN-infected birds had viremia that lasted 5 or 6 days (Table 4) and achieved a mean peak virus titer of 5.4 $\log_{10}$ PFU/ml. In contrast, none of the geese that received a 10-times higher dose of the WN/DEN4 or WN/DEN4Δ30 virus showed any evidence of detectable viremia or developed WN-specific neutralizing antibodies, indicating an inability of these viruses to replicate and to stimulate an immune response in birds. As a result, the chimeric viruses failed to protect geese from replication of WN virus following challenge and from morbidity attributed to WN infection, indicating that chimeras are not useful as a vaccine for birds. However, an important conclusion of this study is that if WN/DEN4 or WN/DEN4Δ30 were to be used as a vaccine for human or horses, the vaccine virus would not be amplified in WN-competent avian hosts and, therefore, could not be introduced into the natural transmission cycle.

Over the past 6 years WN has spread across North America and, more recently, has extended its range to include the Caribbean islands and Latin America, geographic regions in which the four serotypes of dengue virus are endemic. If WN/DEN4Δ30 virus is used in Central or South America as a vaccine against WN virus, it will have to be immunogenic in individuals with previous dengue virus exposure. It is possible that immunity to dengue viruses might restrict replication of WN/DEN4Δ30 and thereby decrease its immunogenicity. Alternatively, it is possible that previous infection with dengue viruses might enhance the replication of WN/DEN4Δ30 and increase its reactogenicity. Therefore, a study in rhesus monkeys was initiated to determine whether pre-existing immunity to the dengue viruses would affect safety and immunogenicity of WN/DEN4 virus vaccine candidates. Both chimeric viruses were safe in normal monkeys as well as in monkeys having immunity to dengue viruses (Table 6). Pre-existing DEN-immunity did not enhance the replication of either chimeric virus. None of the four monkeys with or without immunity to DEN4 developed a detectable viremia when they were inoculated with the clinical lot of WN/DEN4Δ30 vaccine virus. In addition, both groups of monkeys inoculated with the unmodified WN/DEN4 virus developed a brief, low-level viremia. Since the level of replication of WN/DEN4 and WN/DEN4Δ30 was low in naive monkeys, it was not possible to determine if pre-existing DEN immunity significantly interfered with replication of either chimeric virus. This possibility was addressed indirectly by comparison of the antibody response to WN in both DEN-immune and non-immune monkeys.

Following WN/DEN4 or WN/DEN4Δ30 inoculation, 100% of monkeys seroconverted to the WN. Levels of WN neutralizing antibody titers appeared to be similar across groups of monkeys with or without DEN immunity. However, a two-fold decrease in mean WN antibody titer was seen in DEN-immune compared to non-immune monkeys suggesting that previous immunity to dengue viruses resulted in a slight decrease in the immunogenicity of the chimeric WN vaccine. Clearly, enhancement of replication was not supported by the immunization data. It is possible that cross-reactive antibodies or cross-reactive cellular immunity to dengue viruses acted to restrict replication of the WN/DEN4 chimeric virus and, therefore, decrease its immunogenicity. It was shown previously [29–31] that antibodies against the flavivirus nonstructural protein NS1 can protect against infection *in vivo*. In the present study, the effect of cross-reactive immunity to DEN is a very small and does not significantly interfere with the development of neutralizing antibodies to WN. There was a correlation between the magnitude of serum WN-specific antibody response induced by WN vaccine immunization and the degree of virus replication in monkeys, i.e., the unmodified WN/DEN4 chimera that caused a brief viremia in both DEN-immune and naive monkeys was more immunogenic than its highly attenuated Δ30 mutant. The decreased immunogenicity of the clinical lot of WN/DEN4Δ30 was consistent with its failure to induce a detectable viremia in DEN-immune monkeys and naïve monkeys observed in this study (Table 6) and previously for the experimental lot of WN/DEN4Δ30 [5]. Since the experimental lot provided complete protection in rhesus monkeys against wild-type WN virus challenge [5] and since the two lots of vaccine exhibited comparable immunogenic-

ity, the protective efficacy of the clinical lot was not evaluated in present study since. Thus, there was no significant difference in the level of replication (viremia) or in the level of the induced WN-specific neutralizing antibodies between DEN-immune monkeys and naïve monkeys vaccinated with a single dose of WN/DEN4 or WN/DEN4Δ30. Based on these observations, it is expected that pre-existing immunity to the dengue viruses will not be a factor limiting the practical utilization of chimeric WN vaccine in humans living in the regions that are endemic for dengue viruses.

Most of the DEN-immune monkeys responded to WN/DEN4 or WN/DEN4Δ30 immunization with an increase in neutralizing antibody titer against DEN4, suggesting that this anamnestic response could be induced by shared antigenic determinants between DEN4 and chimeric viruses. It was shown previously that antigenic determinants of flavivirus cross-reactive antibodies have been mapped to domain II of structural protein E that contains a fusion peptide sequence, which is conserved among the flaviviruses. Monoclonal antibodies with binding sites at or near the fusion peptide have broad cross-reactivity with flaviviruses including DEN4 and WN [32,33].

In summary, the clinical lot of the more attenuated vaccine candidate, a WN/DEN4Δ30 virus, exhibited a satisfactory balance between attenuation and immunogenicity in mice and monkeys. The lack of infectivity for geese, the reduced infectivity for mosquitoes, and the low level of replication of WN/DEN4Δ30 virus in non-human primates suggest that this vaccine candidate would not be transmitted from vaccinees to other hosts in the environment and would not be maintained in those hosts. Currently, a phase I clinical trial in humans is underway with the WN/DEN4Δ30 vaccine.

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